JOURNAL OF CLINICAL MICROBIOLOGY, May 1992, p. 1085–1088
0095-1137/92/051085-04$02.00/0
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Vol. 30, No. 5

Multicenter Evaluation of a New Enzyme Immunoassay for Detection of *Clostridium difficile* Enterotoxin A

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Received 8 November 1991/Accepted 10 February 1992

The Premier *Clostridium difficile* toxin A enzyme immunoassay (PTA EIA) (Meridian Diagnostics, Inc., Cincinnati, Ohio) for rapid diagnosis of antibiotic-associated colitis (AAC) was evaluated in a multicenter study. Stool samples from 421 patients suspected of having AAC were tested for toxin A by the PTA EIA and for toxin B by three tissue culture assays (TCA) employing WI-38 cells (New England Deaconess Hospital) in conventional tubes or foreskin fibroblasts (Children's Hospital) or Vero cells (Beth Israel Hospital) in microwells. The tubes and plates were examined at 24 and 48 h for cytotoxicity. Clinical criteria, repeat testing at another site, and culture of frozen stool samples for *C. difficile* were used to evaluate discrepant results. Of 504 samples, 66 were positive and 409 were negative by both tests. Eight samples had indeterminate PTA EIA results and were excluded from this analysis. Of 21 discrepancies, 9 were PTA EIA positive and TCA negative and 12 were PTA EIA negative TCA positive. Following resolution of the discrepancies, 11 of 12 PTA EIA-negative–TCA-positive and 5 of 9 PTA EIA-positive–TCA-negative samples were considered true positive for AAC. The sensitivity and specificity were, respectively, 86.6 and 99.0% for the PTA EIA and 93.9 and 99.8% for TCA. The predictive values of positive and negative tests were, respectively, 94.7 and 97.4% for the PTA EIA and 98.7 and 98.8% for TCA. We conclude that the PTA EIA is a rapid, simple EIA technique whose accuracy in detecting enterotoxin A approaches that of reference TCA methods for detection of cytotoxin B.

Clostridium difficile is the most important cause of antibiotic-associated diarrhea and colitis (10). Nosocomial transmission of the organism is frequent and difficult to control, and disease due to *C. difficile* contributes significantly to the morbidity and length of stay of many hospitalized patients (14).

Diagnostic methods for *C. difficile* are based on either isolation and characterization of the organism or direct detection of toxins or bacterial cell wall antigens in stool (10). Culture methods, although very sensitive, require fairly lengthy processing and additional testing of the isolates to determine toxigenicity, which tends to limit their use for diagnostic purposes. Of the direct detection methods, the most established is a cytotoxicity assay (tissue culture assay) for cytopathic toxin B first described by Chang and colleagues in 1978 (5). This assay, although sensitive and specific, requires experience with tissue culture techniques and up to 48 h of incubation. Rapid latex agglutination methods which detect a nontoxic bacterial antigen are now available. These tests are positive with both toxigenic and nontoxigenic strains of *C. difficile* and therefore may require confirmation of positive results by other methods (8, 15, 21). Until recently, immunologic detection of enterotoxin A, a pathogenetically important toxin, was hampered by the unavailability of reliable antibodies. Monoclonal and polyclonal antibodies to enterotoxin A for use in an enzyme immunoassay (EIA) were developed in the early and mid 1980's by Wilkins and colleagues (11, 12). Several rapid EIAs for detection of enterotoxin A have since become commercially available.

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This report describes a multicenter evaluation of a premarket formulation of the Premier *C. difficile* toxin A (PTA) EIA (Meridian Diagnostics, Inc., Cincinnati, Ohio) conducted at three teaching hospitals. Patients included both adults and children suspected of having antibiotic-associated diarrhea or colitis.

(This study was presented in part at the 91st General Meeting of the American Society for Microbiology, Dallas, Tex., May 1991.)

MATERIALS AND METHODS

PTA EIA. The PTA EIA utilizes plastic breakaway wells coated with polyclonal antibodies to *C. difficile* toxin A (capture antibody) and an enzyme-conjugated monoclonal antibody to toxin A (detector antibody). The assay was performed in accordance with the manufacturer's instructions. Briefly, stool was diluted 1:5 (50 μl of stool in 200 μl of sample diluent) and 1 drop was added to each well together with 1 drop of monoclonal antitoxin A conjugated to horseradish peroxidase. After 2 h of incubation at 35°C, the plate was washed five times manually, after which 1 drop each of urea peroxide (substrate A) and tetramethylbenzidine (substrate B) was added to the wells. After 10 min of incubation at room temperature, 1 drop of sulfuric acid (stop solution) was added and the plate was shaken for 15 s.

Reading was performed both visually and spectrophotometrically within 15 to 30 min of addition of the stop solution. Visually, a colorless well was interpreted as negative, a faintly yellow well was indeterminate, and a definitely yellow well was positive. The absorbance values indicated by the manufacturer were used for spectrophotometric interpretation. A single-wavelength spectrophotometer blanked

to air and set at 450 nm was used at Beth Israel Hospital and Children's Hospital, and a dual-wavelength spectrophotometer blanked to air and set at 450 and 630 nm was used at New England Deaconess Hospital (NEDH).

Cytotoxin assays. The cell lines used included WI-38 human lung fibroblasts in conventional tubes (M.A. Bioproducts, Walkersville, Md.) at NEDH, human foreskin fibroblasts in microwells (Bartels cytotoxicity assay; Baxter Healthcare Co., West Sacramento, Calif.) at Children's Hospital, and Vero cells in microwells prepared by the virology section at Beth Israel Hospital.

Testing protocols were similar and included a dilution step in phosphate-buffered saline, a centrifugation and/or filtration step, and inoculation of stool supernatant onto the monolayers. The inoculum sizes were 100 μl for the WI-38 cell assay in conventional tubes, 50 μl for the Bartels kit microassay, and 20 μl for the Vero cell microassay, yielding final dilutions of 1:30, 1:40 and 1:80, and 1:30, respectively.

All cell lines were examined at 24 and 48 h of incubation at 35°C. Samples were deemed positive by cytotoxin assay if a characteristic cytopathic effect was observed and the cytopathic effect was neutralized by antitoxin addition. Antitoxin was obtained from Bartels, Baxter Healthcare Co., and Tech-Lab Inc. (Blacksburg, Va.).

Culture for *C. difficile*. All frozen aliquots of specimens yielding discrepant results or spectrophotometrically indeterminate PTA EIA results were cultured for *C. difficile*.

After being thawed at room temperature, stool was inoculated directly onto a plate of cycloserine-cefoxitin-fructose agar containing sodium taurocholate (formulation of Wilson et al. [23]) prepared at NEDH. A second cycloserine-cefoxitin-fructose agar plate was inoculated with stool which had been heated in a water bath at 80°C for 10 min. The remainder of the heated stool was mixed with an equal volume of 1 M thioglycolic acid adjusted to pH 10, further heated in a water bath at 50°C for 30 minutes (4), and inoculated to a third cycloserine-cefoxitin-fructose agar plate and an anaerobic blood agar plate prepared at NEDH.

Plates were incubated in anaerobic jars (Oxoid gas-generating kit; Unipath Ltd., Basingstoke, England) for 48 h at 35°C. Colonies exhibiting characteristic morphology and odor were identified by biochemical characterization (Rap IDS Ana II System; Innovative Diagnostic Systems Inc., Atlanta, Ga.). All isolates were grown in prereduced anaerobically sterilized brain heart infusion broth, and supernatants were assayed for production of toxins A and B.

Study design. (i) Initial testing. A total of 504 stool specimens from 421 adults and children were studied. All samples were submitted to each of the teaching hospital laboratories for detection of *C. difficile* cytotoxin B. Stools were stored at 4°C and tested in parallel by cytotoxicity assay and PTA EIA within 48 h of collection. Readings were conducted without prior knowledge of the other method's result. Specimens submitted within 72 h of receipt of an initial sample were excluded from the study.

(ii) Analysis of discrepant results. Aliquots of specimens yielding discrepant results by cytotoxicity and PTA EIA or spectrophotometrically indeterminate results by PTA EIA were stored at −70°C for further testing. All such frozen aliquots were retested by two cytotoxicity assays, including the one originally performed, and by PTA EIA at one of the other hospitals and cultured for *C. difficile* at NEDH. To ensure the adequacy of our methods, some specimens positive for *C. difficile* enterotoxin A and cytotoxin B were frozen and tested by using the methods described here.

The charts of patients with specimens yielding discrepant or indeterminate results were reviewed. Patients were categorized as likely to have antibiotic-associated diarrhea or colitis if all of the following criteria were met: (i) diarrhea (six or more liquid stools over a 36-h period), (ii) recent antibiotic use (within 8 weeks of onset of diarrhea), (iii) pseudomembranous colitis at endoscopy or no other demonstrable cause for diarrhea, (iv) response to oral vancomycin or metronidazole if administered. Specimens yielding discrepant results were categorized as true positive for *C. difficile* colitis in the presence of a positive clinical history associated with one or more positive laboratory tests (either positive culture yielding a toxigenic strain or a repeatably positive test for cytotoxin B or enterotoxin A).

TABLE 1. PTA EIA compared with cytotoxicity assays

PTA EIA result	No. with the following cytotoxicity assay results	
	Positive	Negative
Positive	66	9
Negative	12	409
Indeterminate	3	5

RESULTS

Of 504 specimens tested, 496 could be evaluated (Table 1); 66 were positive by both cytotoxicity assay and enterotoxin A EIA, and 409 were negative by both methods. Furthermore, there were 21 specimens with discrepant results and 8 (1.6%) with spectrophotometrically indeterminate results for toxin A. For determining the sensitivity and specificity of the toxin A EIA, these eight were excluded from analysis.

The 21 specimens yielding discrepant results were further studied by repeat toxin A EIA and cytotoxicity assay at another institution, by culture for *C. difficile*, and by review of the patients' clinical histories. Of 12 specimens positive by cytotoxicity and negative by toxin A EIA, 11 were considered true positive and 1 was true negative (Table 2). The latter sample, which originally tested positive for cytotoxicity with the Bartels assay, was retested with both the Bartels assay and another assay (WI-38 cells). Both methods gave negative results for cytotoxicity. Of nine specimens positive by toxin A EIA and negative by cytotoxicity, four were considered true negative and five were true positive (Table 3). Repeat testing of the five true-positive samples which originally tested positive for toxin A and negative for cytotoxin yielded three positive and two negative results for cytotoxin, as well as one indeterminate and four positive results for toxin A. Of the four true-negative samples which originally tested positive for toxin A and negative for cyto-

TABLE 2. Analysis of discrepant results obtained with PTA EIA-negative and cytotoxicity-positive samples[a]

No. of specimens	Repeat testing result		Culture result	History
	PTA EIA	Cytotoxicity		
6	Neg	Pos	Pos	Pos
2	Neg	Pos	Neg	Pos
1	Pos	Pos	Pos	Pos
1	ND	ND	Pos	Pos
1	Neg	Neg	Pos	Pos
1	Neg	Neg	Neg	Neg

[a] Neg, negative; Pos, positive; ND, not done.

TABLE 3. Analysis of discrepant results obtained with PTA EIA-positive and cytotoxicity-negative samples[a]

No. of specimens	Repeat testing result		Culture result	History
	PTA EIA	Cytotoxicity		
3	Pos	Pos	Pos	Pos
3	Neg	Neg	Neg	Neg
1	ND	ND	Neg	Neg
1	Pos	Neg	Neg	Pos
1	Ind	Neg	Pos	Pos

[a] Neg, negative; Pos, positive; ND, not done; Ind, indeterminate.

toxin, only three could be retested. All three gave negative repeat results for both toxin A and cytotoxin. After resolution of the discrepancies, there were 11 false-negative and 4 false-positive results by toxin A EIA, as well as 5 false-negative results and 1 false-positive result by cytotoxicity assay ($P > 0.05$ by the χ^2 test). Sensitivity, specificity, predictive values (positive and negative), and efficiency of both toxin A EIA and cytotoxicity assays following resolution of discrepancies, are shown in Table 4.

The eight specimens yielding spectrophotometrically indeterminate results with toxin A EIA were also subjected to further testing and review. Repeat toxin A EIA of the frozen aliquots at another study hospital yielded one positive and seven negative results. In four of the eight specimens, toxigenic *C. difficile* was isolated and all four patients had clinical histories suggestive of antibiotic-associated diarrhea. Three of these specimens were also repeatably positive for cytotoxin B. The remaining four had negative clinical histories. Furthermore, their stool samples were cytotoxin B negative although two yielded toxigenic *C. difficile* by culture. Thus, of the eight specimens, four were considered true positive and four were true negative.

There were no major discrepancies when visual versus spectrophotometric readings of toxin A EIA were compared (Table 5). All specimens interpreted as positive or negative visually were similarly positive or negative spectrophotometrically, except for four visually negative, spectrophotometrically indeterminate samples. However, visual reading yielded 20 indeterminate results. The corresponding spectrophotometer results showed 10 positive, 6 negative, and 4 indeterminate results. Review of clinical data for these patients and additional testing by culture and cytotoxin B detection on frozen aliquots showed that 10 of 20 had *C. difficile*-associated diarrhea. It is of note that 9 of these 10 true-positive specimens were identified as positive by spectrophotometer readings and 1 was indeterminate. Additionally, four specimens were indeterminate by spectrophotometry but visually negative. Of these, three had *C. difficile*-associated diarrhea by history and other testing.

TABLE 4. Performance of PTA EIA and cytotoxicity assay after resolution of discrepancies

Parameter	PTA EIA	TCA[a]
Sensitivity (%)	86.6	93.9
Specificity (%)	99.0	99.8
PVP[b] (%)	94.7	98.7
PVN[c] (%)	97.4	98.8
Efficiency (%)	97.0	98.8

[a] TCA, tissue culture assays.
[b] PVP, predictive value of a positive result.
[c] PVN, predictive value of a negative result.

TABLE 5. Visual versus spectrophotometric readings of PTA EIA

Spectrophotometric result	No. with the following visual results		
	Positive	Negative	Indeterminate
Positive	64	0	10
Negative	0	416	6
Indeterminate	0	4	4

DISCUSSION

Laboratory methods used for detection of *C. difficile* toxin include tissue culture, counterimmunoelectrophoresis, latex agglutination (LA), and EIA. While the tissue culture assay has been considered the "gold standard" because of its high sensitivity (detecting <1 pg of toxin B) and specificity (10), it requires expertise in tissue culture techniques and up to 48 h of incubation for a final result.

The search for rapid techniques has evolved from counterimmunoelectrophoresis assays developed in the early 1980's, which suffered from lack of both specificity and sensitivity (18, 20, 22, 24), to LA and more recently to EIA methods, which have become commercially available and are gaining popularity because of their rapidity and ease of performance.

LA methods were first thought to detect toxin A as characterized by Banno et al. (1, 2). Subsequently, however, they were shown to detect a nontoxic bacterial antigen present in toxigenic and nontoxigenic strains of *C. difficile*, *C. sporogenes*, *C. botulinum*, and other anaerobic bacteria (3, 7, 13). Evaluations of commercially available LA assays have found widely ranging sensitivities and specificities (6, 8, 16, 17, 19, 21). Some investigators have concluded that LA tests, while less sensitive than cytotoxicity, are acceptable for screening, provided that positive results are confirmed by another method (17, 21).

EIA methods have been used for detection of enterotoxin A and cytotoxin B (9, 12), but these have not been commercially available until recently. This study evaluated the performance of a new rapid EIA (PTA EIA [Meridian Diagnostics, Inc.]) in comparison with commercial and noncommercial versions of the cytotoxicity assay for diagnosis of *C. difficile*-associated diarrhea or colitis. The method, which detects toxin A in stool, is technically easy and is completed in 2.5 h. The evaluation was performed at three teaching hospitals and included adults and children suspected of having antibiotic-associated colitis. Discrepancies were resolved by repeat testing and culture and review of clinical histories. The PTA EIA performed almost as well as the cytotoxicity assays, with specificity, positive and negative predictive values, and efficiency all ≥95%.

The sensitivity for the EIA was 86.6%, compared with 93.9% for cytotoxicity. These results were obtained by using spectrophotometric rather than visual readings of the assay. While visual reading resulted in no major discrepancies compared with spectrophotometry, it gave rise to a higher number of indeterminate results (20 compared with 8). This was likely due to the subjectivity inherent in categorizing light and very light shades of yellow in the wells. This was found to be true even though interpretation of the results was performed by only one or two technologists at each institu-

tion. Therefore, we recommend the use of a spectrophotometer (single or dual wavelength) for reading of the assay's results in a clinical laboratory setting.

Specimens yielding repeatedly indeterminate results by spectrophotometry (1.6% in this study) cannot not be dismissed as negative and should be tested by another method (i.e., cytotoxicity or culture). This is best illustrated by the fact that in this study, of eight initially indeterminate results, three specimens were positive by cytotoxicity, four patients had clinical histories suggestive of *C. difficile*, and six samples yielded toxigenic *C. difficile* in culture.

Although washing of the plates was performed manually during this study, use of an automated plate washer is recommended to reduce the risk of splashing potentially hazardous material when the stool suspension is first discarded from the wells.

In conclusion, the premarket formulation of the PTA EIA can be used as an adjunct to cytotoxicity or as an alternative to it, provided that samples yielding indeterminate results are referred to another laboratory for further testing. It should be particularly valuable as a rapid screen in laboratories handling a moderate-to-high volume of tests and whenever same-day reporting is deemed essential.

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